Hyphenation of a Deoxyribonuclease I immobilized enzyme reactor with liquid chromatography for the online stability evaluation of oligonucleotides

Piotr Wiktor Álvarez Porebski[1], Ellen Gyssels[2], Annemieke Madder[3], Frederic Lynen[4]*

[1,4] *Separation Science Group, Department of Organic and Macromolecular Chemistry, Universiteit Gent, Krijgslaan 281 S4-bis, 9000 Gent, Belgium*

[2,3] Organic and Biomimetic Chemistry Research Group, *Department of Organic and Macromolecular Chemistry, Universiteit Gent, Krijgslaan 281 S4-bis, 9000 Gent, Belgium*

[1] PiotrWiktor.AlvarezPorebski@ugent.be

[2] Ellen.Gyssels@ugent.be

[3] Annemieke.Madder@ugent.be

[4] Frederic.Lynen@UGent.be

Tel: +32 (0)9 264 9606

Fax: +32 (0)9 264 4998

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Abstract

The stability of antisense oligonucleotides (ONs) towards nucleases is a key aspect for their possible implementation as therapeutic agents. Typically, ON stability studies are performed off-line, where the ONs are incubated with nucleases in solution, followed by their analysis. The problematics of off-line processing render the detailed comparison of relative ON stability quite challenging. Therefore, the

development of an online platform based on an immobilized enzyme reactor (IMER) coupled to liquid chromatography (LC) was developed as an alternative for improved ON stability testing. More in detail, Deoxyribonuclease I (DNase I) was immobilized on epoxy-silica particles of different pore sizes and packed into a column for the construction of an IMER. Subsequently, the hyphenation of the IMER with ion-pair chromatography (IPC) and ion-exchange chromatography (IEC) was evaluated, leading to the successful development of two online methodologies: IMER-IPC and IMER-IEC. More specifically, natural and modified DNA and RNA oligonucleotides were used for testing the performance of the methodologies. Both methodologies proved to be simple, automatable, fast and highly reproducible for the quantitative and qualitative evaluation of ON degradation. In addition, the extended IMER life time in combination with a more straightforward control of the reaction kinetics substantiate the applicability of the IMER-LC platform for ON stability tests and its implementation in routine and research laboratories.

1. Introduction

In the last two decades the therapeutic potential of antisense oligonucleotides (ONs) has been firmly recognized. These single-stranded short nucleic acid chains are designed to hybridize complementary sequences in DNA, pre-mRNA and mRNA, and in this way, block the expression of a target gene [1-3]. The main problems related with the development and implementation of therapeutic ONs include their intracellular delivery, transfection across the membrane and stability against nucleases [2, 3]. In an attempt to improve upon the latter, many modifications on the phosphodiester backbone, the sugar or the base moiety have been described for increasing nuclease resistance [2, 3]. For instance, the phosphorothioate modification is one of the simplest chemical modifications which can be carried out in the ON [3, 4]. Hereby a non-bridging oxygen of the phosphorus is substituted by a sulfur atom.

Phosphorothioates have demonstrated high resistance towards nucleases; however, their biggest drawback is the reduced ON hybridizing ability towards complementary strands [3, 4]. Therefore, many other chemical modifications and their combinations have been considered in order to seek for an optimal balance between the hybridization ability of the ONs and their resistance towards nucleases [3-5].

Stability studies of ON have been mainly performed in vitro [4, 6-24]. For this purpose ONs are typically incubated with a specific nuclease, mixture of nucleases, biological fluid or tissue homogenates, followed by their analysis in various ways. The initially developed analysis techniques required the usage of fluorescent or radiolabeled ONs in combination with polyacrylamide gel electrophoresis (PAGE) [6-8, 10, 14, 15, 25] or thin layer chromatography (TLC) [6]. However, those procedures were laborious, often lacking good sensitivity, and attaining accurate and precise quantitation results remains challenging. Afterwards, with the increasing use of capillary electrophoresis (CE) [23, 24, 26-28] and liquid chromatography (LC) [6, 29], labeled ONs were no longer required. The usage of CE has proven to deliver a base to base resolution for ONs differing by one nucleotide in length. Moreover, the substitution of polyacrylamide filled capillaries by entangled polymer solutions drastically improved the reproducibility of the methodology and facilitated its implementation [27, 30]. Nevertheless, the high ionic strength of the enzymatic incubation media, requires a desalting step of the sample for an adequate CE analysis [23, 24, 26, 27, 31], complicating in this way the sample manipulation procedure, affecting the analysis time and reproducibility of the methodology. In contrast, the greater robustness of LC, in addition to recent advances in the development of higher efficiency columns which are able to provide a high resolution separation of ONs in the range of 10-*mer* to 30-*mer* [29, 30, 32], made this technique the preferred candidate for ON stability studies in pharmaceutical environments [9, 11, 13, 16, 18, 20-22]. Among the existing LC techniques, ion-pair chromatography (IPC) and ion-exchange chromatography (IEC) have proven to be suitable for this purpose [20, 24, 29, 30, 33]. Additionally, ON

separations by IPC and IEC (with a polymeric support), do not occur exclusively based on the length of the ON, but also depend on the base composition [32]. Thus, discerning between two ON product fragments which have the same length but a significantly different base composition can be efficiently achieved using either of these two techniques [30]. Analytical procedures employed thus far for ON stability share in common the presence of enzymes in solution, for which the reaction is performed by batch incubation [4, 6, 9-11, 13-16, 18, 19, 25-27] or in online way [34]. Incubation can be performed using either the enzyme present in solution or immobilized on a solid support. Enzyme immobilization offers several advantages when compared to enzymatic reactions carried out in solution. In addition to an easier separation of the enzyme from the reaction products and/or substrate, the immobilization of enzymes also confers them an increased stability towards mechanical stress, pH, heat, ionic strength and organic solvents [35-41]. Moreover, once packed into the columns to be used as immobilized enzyme reactors (IMERs), they can be employed in a continuous operation mode, reducing in this way the sample manipulation steps, increasing the reproducibility and allowing their reutilization; therefore, reducing costs and analysis time.

The immobilization of enzymes on solid supports has been widely studied and numerous immobilization methods and supports which are commonly employed have been reported in literature [35, 36, 38, 40-43]. Several nucleases are commercially available, and a broad variety of combinations can be tested based on the type of substrate they hydrolyze (DNA or RNA), the type of nucleophilic attack (exonuclease and/or endonuclease), the nature of the hydrolytic products (mono or oligonucleotides) and the nature of the bond which is hydrolyzed [44, 45]. Furthermore, factors such as their biological relevance, kinetic performance and cost, also need to be considered for their application in IMERs.

Deoxyribonuclease I (DNase I), is a 29.1 kDa endonuclease which cleaves the phosphodiester backbone of double and single stranded DNA, requiring divalent cations as cofactors [46, 47]. The enzyme cleaves the P-O3’ bond of the DNA backbone, yielding 5’-phosphate oligonucleotides [47-49]. Moreover, DNase I

has been used as a powerful footprinting agent and for DNA probing [50, 51]. The immobilization of DNase I on monolithic supports [52, 53], polymers [54] and magnetic particles [55] has been reported. Furthermore, the good catalytic performance of the immobilized endonuclease and its low cost, makes DNase I a good candidate for its implementation in IMERs.

In this study, we describe the construction of an IMER with bovine DNase I, and the evaluation of its hyphenation with IPC and IEC using natural and modified DNA and RNA ONs. Two online platforms (IMER-IPC and IMER-IEC) with potential applications in ON stability studies were successfully developed. To the best of our knowledge, the development of online IMER-LC methodologies for stability testing of ONs had not been reported yet, and could be of high relevance in the development of improved antisense ON therapies.

2. Experimental

2.1 Chemicals

Triethylamine (TEA), acetic acid, adenosine monophosphate (AMP), hydrochloric acid, chloroform, Tris, NaCl, acetone (HPLC grade), Na2HPO4, EDTA, CaCl2, MgCl2, glycine, (3-Glycidyloxypropyl) trimethoxysilane (GPTMS) and Deoxyribonuclease I (DNase I) from bovine pancreas (≥400 Kunitz units/mg of protein) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (ACN) HPLC grade from Fischer Scientific (Loughborough, U.K.) and Milli-Q water (Milipore, Milfold, MA) were used. All the mobile phases were filtered through 0.22 µm nylon membrane filters (Grace Davison Discovery Sciences, Lokeren, Belgium). Homo-oligonucleotides of deoxythymidine 15-*mer* (T15), 20-*mer* (T20) and 30-*mer* (T30), deoxyadenosine 30-*mer* (A30), 12-*mer* ON (5'-GCA-CAC-CGT-CAG-3') and a 41-*mer* ON (5'-GTT-GGA-TTA-AAC-AAC-CGT-TCC-CGT-CTC-TAT-CAG-CTT-AGT-GT-3') all based on the phosphodiester backbone; 15-*mer* (5'-T*T*T*-T*T*T*-T*T*T*-T*T*T*-T*T*T*-3') with a phosphorothioate backbone,

and the 2' O-methyl RNA 12-*mer* ON (5'-GCA-CAC-CGU-CAG-3') were purchased from Eurogentec (Liege, Belgium). The lyophilized ONs were dissolved in an appropriate volume of Mili-Q water in order to obtain 100 µM stock solutions.

2.2 Instrumentation

An Agilent 1100 series HPLC composed of a binary pump and a single wave length detector was employed for the delivery of the chromatographic mobile phases, while the enzymatic reaction buffer was delivered to the reactor by a HP 1050 quaternary pump (Agilent Technologies, Waldbronn, Germany). A CTO-20AC prominence HPLC column oven (Shimadzu, Kyoto, Japan) was used for controlling the temperature of the IMER.

2.3 Preparation of epoxy-functionalized silica

Nucleosil 5 µm spherical silica particles (1.2 g) of 300, 1000 and 4000 Angstrom from Macherey-Nagel (Duren, Germany) were used. The functionalization of the silica particles was carried out by suspending them in a 10% (v/v) GPTMS solution in acetone at room temperature for 2 h under intense stirring. Afterwards, the particles were filtered and thoroughly washed with acetone, followed by phosphate buffer (pH 7.0; 15 mM) containing 0.1 M NaCl and finally with phosphate buffer [56]. The particles were dried at 37 ℃ and then stored at 4 ℃ until the enzyme immobilization was carried out.

2.4 Immobilization of DNase I

Three mL of a 5 mg/mL solution of DNase I in phosphate buffer (pH 7.0; 15 mM) was mixed with 1 g of the functionalized silica particles and stirred at 3000 rpm for 30 min. Subsequently, the mixture was set

in the fridge (4 ℃) for 12 h. When the immobilization was completed, the enzyme modified silica was filtered and washed with phosphate buffer. Afterwards, deactivation of the unreacted epoxy groups was carried out by mixing the particles with a 0.2% (m/v) glycine solution in phosphate buffer and stirring for 2 h at room temperature. Once the deactivation was completed, the particles were filtered and washed with phosphate buffer containing 0.1 M NaCl, followed by phosphate buffer and then by the reaction buffer (pH 7.5; 50 mM Tris, 5 mM $CaCl_2$ and 5 mM $MgCl_2$). Finally, the enzyme modified silica particles were dried at 37 ℃ and stored at 4 ℃ before use. The amount of enzyme bound to the silica particles was determined from the concentration difference of the DNase I solution before and after the immobilization using flow injection analysis at 280 nm.

2.5 Preparation of the IMERs

150 mm x 2.1 mm I.D. stainless steel columns were packed using 5 mL slurry containing 1g of enzyme-silica particles in phosphate buffer (pH 7.0; 15 mM). The slurries where previously homogenized for 10 minutes in an ultrasonic bath and consequently packed in the columns at a constant pressure of 400 bar for 2h using the phosphate buffer as packing solvent.

2.6 Kinetic determinations

The biological activity of DNase I was determined by measuring the degradation that a deoxythymidine 30-*mer* oligonucleotide (T30) experienced under the incubation with this enzyme. For this, Ion-pair chromatography (IPC) using a XBridge C18 column 50 mm × 4.6 mm I.D. × 3 µm column (Waters, Zellik, Belgium) was used, and the separation temperature was maintained at 60 ℃. The IP mobile phase was composed of 100 mM TEA (pH 5.5 adjusted with acetic acid) (A) and ACN (B). The gradient profile consisted of 0-50 min, 0-16B% and the detection was performed at 260 nm. A calibration curve for the

T30 ON (0.05-60 μM) was constructed for calculating the amount of T30 in the blank and in the test samples. The enzyme activity was expressed as the pmol of T30/min degraded under the assay conditions described in sections 2.6.1 and 2.6.2.

2.6.1 Activity of free DNase I

A T30 ON (final concentration 1-50 μM) was incubated with DNase I (0.01-0.0005 mg/mL) in 50 mM Tris buffer pH 7.5, 5 mM $CaCl_2$ and 5 mM $MgCl_2$, at 37 ℃ (final volume of 100 μL) and stirred at 2000 rmp. The incubation time ranged from 0.5-5 min and the reaction was stopped by adding 10 μL of 0.5 M EDTA solution. Afterwards, the sample was analyzed by IPC as described in section 2.6. The reaction rate (μM T30/min) was calculated at the beginning of the reaction in the linear part, and the results were used to construct the Michaelis–Menten plots [37]. The specific activity was calculated by dividing the enzyme activity by the amount of dissolved enzyme. Non-linear regression analysis was performed for calculating the kinetic parameters.

2.6.2 Activity of immobilized DNase I

The activity of the immobilized DNase I was determined in batch in order to attain similar reaction conditions to the assay in section 2.6.1. The immobilized DNase I silica particles (10-1 mg) were incubated with a T30 ON (final concentration 1-50 μM) in 50 mM Tris buffer pH 7.5, 5 mM $CaCl_2$ and 5 mM $MgCl_2$, at 37 ℃ (100 μL) and stirred at 2000 rpm. The vials were incubated for a period ranging from 5-90 min followed by the addition of 10 μL of 0.5 M EDTA solution in order to stop the reaction. Afterwards, the solutions were filtered through 0.45 μm syringe filters before their instrumental analyses. The kinetic parameters were determined as described in section 2.6.1. The specific activity was calculated by dividing the enzyme activity by the amount of immobilized enzyme.

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187 *2.7 Online IMER-LC setup*

188 The employed instrumental setup is depicted in figure 1. This fully automated analytical procedure can
189 be subdivided in two main events: the reaction and focusing of the analytes (i) and the separation (ii).
190 Table 1 resumes the chromatographic conditions of the integrated IMER-LC system.

191 (i) Pump 1, which delivered de reaction buffer, was directly connected to the HP 1100 auto-
192 injector (1-5 µL of injection volumes were used), followed by the IMER which was kept at
193 37 ℃ during all the experiments. By changing the flow rate passing through the reactor, the
194 residence time of the sample could be tuned in order to attain the desired degradation rate
195 for the ONs. The system's and reactor's dead volumes were determined by injecting a 1 mM
196 caffeine solution. The effluent from the bioreactor was mixed with the LC mobile phase
197 (pump 2) with the aid of a T-piece. In this way, both the initial chromatographic mobile
198 phase and the reaction buffer, were continuously delivered to the LC column. This
199 composition and flow rates were kept constant for sufficient time in order to assure that all
200 the sample plug exited the reactor and focused on the head of the LC column; for instance:
201 for a reaction buffer flow rate of 50 µL/min (pump 1), the LC separation mobile phase was
202 kept at 500 µL/min for 40 min, exceeding by this, on average, 4 times the reactor's dead
203 volume (Table 1).

204 (ii) Two separation modes were explored: ion-pair chromatography (IPC) and ion-exchange
205 chromatography (IEC), and the separation conditions were based on a previously described
206 methodology [30]. The detection wavelength was fixed to 260 nm and the flow rate was set
207 to 500 µL/min for both separation modes. For IPC, the same separation conditions were
208 used as in section 2.6. During the reaction-focusing step, the IPC mobile phase composition
209 was kept at 100 % of A; after which, the separation process took place, for which the

reaction buffer flow rate (pump 1) was automatically lowered to 1 µL/min and the IPC gradient was started (pump 2). The IEC experiments were performed on a polymeric based analytical column 4 mm × 250 mm DNAPac PA200 protected with a 4 mm × 50 mm DNAPac PA200 precolumn (Thermo Scientific, Erembodegem-Aalst, Belgium). The column temperature was set to 25 ℃. The mobile phase consisted of water at pH 11.5 (adjusted with NaOH) (A) and water with 1.25 M NaCl at pH 11.5 (B). The gradient profile was 0–90 min, 0–90%B. The same conditions as in the IPC methodology were set for pump 1.

It must be pointed out that each pump functioned accordingly to its own elution program and that they were interconnected via an analog interface for their synchronization; thus, the start of the analysis was controlled by the *ChemStation* software from the Agilent 1100 instrument.

3. Results and discussion

3.1 Immobilization of DNase I

The first goal was to develop a reactor allowing maximal enzymatic activity while maintaining the residence time to a minimum. Several variables may influence the IMER performance; among them, the choice of the supporting material and the immobilization chemistry of the enzyme to the support, stand out as the most critical. An ideal support should be chemically inert to the reaction conditions and samples. In addition, its physical properties such as porosity, pore size distribution, shape, swelling capacity and charges, play also an important role in the kinetics of the process [35-38]. Spherical silica particles were chosen as support for the enzyme as a variety of pore diameters and particle sizes are available, allowing for example, the optimization of the diffusion process and the pressure drop in the IMER. In addition, their increased resistance to degradation by contaminating microorganisms and a

232 stable morphology towards different solvent conditions, distinct as relevant factors for the development
233 of an IMER [38, 41, 57, 58].

234 Many strategies for immobilizing enzymes have been described, and among the most relevant stand the
235 adsorption and the covalent immobilization [35, 36, 38, 41]. Though covalent immobilization of the
236 enzyme ensures an increased ruggedness and life time of the reactor, one should take into account that,
237 due to the drastic reaction conditions used during the covalent immobilization process, enzymatic
238 activity can be somewhat reduced.

239 A common, if not the most common crosslinking agent for enzyme immobilization is glutaraldehyde [35,
240 36, 38]; however, amino-propyl silica is thereby required as starting material, presenting the
241 disadvantage that ONs strongly adsorb and tail to the unreacted silanol amino functions, rendering this
242 activation process inadequate for the purpose of the here developed methodology. Therefore in this
243 work, the immobilization of DNase I was carried out via nucleophilic attack of the amine groups of the
244 enzyme on the immobilized epoxide groups in silica. Next to the simplicity of this type of silica
245 activation, this procedure results in quasi inert and stable covalent bonds [38, 41, 56, 59]. In addition, no
246 adsorption of the ONs onto the support material could be observed when the glycidyloxypropyl bond
247 chemistry was used as an alternative.

248 As the accessibility of the substrate to the active sites and its diffusivity in the IMER play an important
249 role in kinetic reactions, immobilization on 5 µm silica particles with a pore diameter of 300 Å, 1000 Å
250 and 4000 Å was evaluated. Table 2 shows the amount of enzyme immobilized on the silica supports. It
251 can be noticed that particles with larger pore diameters attach less enzyme in direct correlation with
252 their decreased surface area [60]. This also demonstrates that under the immobilization process
253 conditions, the larger fraction of the enzyme is expected to immobilize close to the pore mouth and on
254 the external surface of the particle rather than deep in the pores [61].

3.2 Activity of free and immobilized DNase

Typically the activity of DNase is determined based on the Kunitz hyperchromicity assay [62], in which the activity of the DNase is manifested as the increase on absorption at 260 nm as a DNA solution is degraded. The substrate used for activity tests has significative effect over the activity of DNase to cleave the phosphodiester backbone. DNase is able to cleave both, single and double stranded DNA, being more active towards this latter one [46]. It has been documented that the sequence also plays an important role, which has been attributed mainly to the flexibility of the DNA [48]. DNA regions rich in A/T nucleotides are rather less flexible and therefore a diminished cleavage is expected [63]. In this study we used a homo-oligonucleotide of deoxythymidine *30-mer* (T30) as substrate, as it will provide a more realistic approach of the DNAse activity towards the goals of the developed methodology, which is to evaluate the stability single stranded oligonucleotides.

The activity of DNase was calculated as the picomoles of T30 degraded per minute. The determination was performed in a batch sequence for both the free enzyme and the immobilized enzyme, to maintain the same incubation conditions, such as the same agitation speed. The activity of the free enzyme was determined as 6.09×10^5 pmol T30/min while the retained activity percentage of the immobilize enzyme was around 0.1% (table 2). The activity decrease upon immobilization is expected, as once immobilized, the enzyme is somewhat restricted and subjected to conformational adaptations in addition to a limited access of the substrate to the active sites. Moreover, it needs to be considered that the covalent immobilization occurs on the multiple regions of the enzyme and that the exposure of the active site might be impeded, therefore the calculated specific activity values are inherently reduced and might be not directly correlated with the immobilized amount. Moreover, additional supports and immobilization techniques might also be explored, what could result in different activities. However, it is important to

point out at this point that by adjusting the flow rate trough the IMER differences in the degree of degradation can be controlled.

By comparing the activity of the immobilized DNase in the 5 µm silica particles of 300 Å, 1000 Å, and 4000 Å it can be noticed that the most active support was the one with an average pore size of 1000 Å (Table 2). On the other hand, the less active support was the one with the smaller pore sizes, despite of a larger amount of immobilized enzyme. This suggests that the larger pore sizes allow for a facilitated interaction of the substrate with active site of the enzyme. This observation is concomitant with degradation profiles of the T30 ON encountered in the IMERs (Figure 2) tested in the developed methodology (section 3.3). Also in Figure 2, it can be noticed that the degradation of the 41-*mer* ON increases as the pore diameter increments, as can be observed in curves A and B in figure 2 corresponding to the IMERs with 1000 Å and the 300 Å pore diameter particles, respectively. The curve of the 4000 Å IMER for the 41-*mer* ON is not presented, as this ON practically degrades in its totality at the fastest residence time tested in our method (~4 min). The higher degradation rate observed for 41-mer ON when compared to the T30 ON is related to secondary structure formation resulting in the presence of a certain degree of duplex structures and also to a mixed nucleotide sequence towards which DNase I is more active [46]. Additionally, a facilitated diffusion of the larger ON through the IMER with the larger pore diameters, also explains this significant differences. Most importantly, the differences in degradation between the ONs are also derived from the DNase I cleavage dependence on the ON sequence [48, 49, 63]. Although further studies on a broader set of ONs could reveal additional factors, at this time it is important to note that this setup satisfactorily allows the comparison and modification of the residence times by adjusting the flow rate.

3.3 Online IMER-LC

The ease of operation, automation and reproducibility of LC when combined with the specificity of an IMER, allows for a highly sensitive methodology to study ON degradation. As in the developed methodologies, the IMER was assembled in the pre-column configuration, the LC separation conditions which could in principle cause possible enzymatic denaturation due to organic solvents, salts and pH of mobile phase, had no effect on the IMER performance. Vice versa, in this setup, the enzymatic reaction conditions should also not alter the performance of the LC separation.

Typically, in pre-column IMER assemblies, both LC chromatographic pumps run independently by the aid of a switching valve [36, 39-41, 64]; nevertheless, in the proposed methodologies, the pumping systems were harmonized and the use of a switching valve was substituted by a T-piece. In this configuration, the IMER effluent was also continuously percolated through the LC column. This increases the simplicity of the methodology; however, the IMER reaction conditions may have an impact over the LC separation performance. Therefore, as first criterion, the IMER reaction conditions must be compatible with the LC methodology. A second possible drawback of this approach is the band broadening, which might be caused due to long residence times in the IMER and non-specific interactions of the analytes with the enzyme and the support material.

As it was required to achieve a high resolution separation of complex ONs mixtures after the reaction, it was critical that the analytes properly focused at the head of the separation column once they emerged from the IMER. As this methodology avoids a switching valve which might allow coupling a trapping column, the LC mobile phase must allow for a proper focusing of the analytes as such.

Ion-pair chromatography (IPC) and Ion-exchange chromatography (EIC) were investigated as those LC methodologies have demonstrated to be suitable for a high resolution separation of therapeutic ONs

[29, 30]. In addition, the IMER reaction buffer (Tris 50 mM, pH 7.5, $CaCl_2$ 5 mM, $MgCl_2$ 5 mM) was compatible with those LC mobile and stationary phases.

3.3.1 Online IMER-IPC

IPC has been widely used to purify ONs; therefore, the efficient focusing effect of the hydrophobic ion-pair formed between the negatively charged ON and triethylamine (TEA) to an octadecyl silane (C18) stationary phase has been documented [29, 33].

Firstly, the focusing of the ONs in IPC under different mixtures ratios of IPC initial mobile phase (TEA 100 mM, pH 5.5) with the IMER reaction buffer was explored in the absence of the IMER and by injecting a poly-T ON mixture under a defined flow rate in pump 1 and pump 2. The conditions were maintained for a defined time, which was established as the time necessary to elute at least 2 to 5 IMER volumes by the flow rate of P1. The tested flow rate ratios (pump 2/pump 1) varied between 2.5 and 20, corresponding to a flow rate ratio pump 2/pump 1 of 500/200 µL/min to 500/25 µL/min, respectively. Those flow rates were mainly dependent on the operational pressure limits in pumps 1 and 2 and the fact that an adequate elution flow rate for the LC separation must be set. Therefore, the flow rate in pump 2 was maintained at 500 µL/min during all the experiments. It must be pointed out that the pressure drop observed in pump 1 with the IMER at a flow rate of 200 µL/min was around 250 bar. In addition, the pressure experienced by pump 1 was also influenced by the pressure drop in pump 2. As consequence, pump 1 was operating near its pressure limit (400 bar) when a flow rate of 500 and 200 µL/min were used in pump 2 and pump 1, respectively. Under the applied conditions before the start of the gradient in pump 2, no breakthrough and satisfactory peak focusing of the ONs was observed. The gradient elution was achieved by programming the pumps in such way that once the focusing was performed, pump 1 automatically reduced the flow rate to 1 µL/min and pump 2 started the gradient program. In

this way the influence of the reaction media over the separation profile was diminished. This was possible as both pumps were programmed independently and as they were interconnected by an analog interface for their synchronization with the LC injector.

Finally, the peak shapes and efficiencies were compared, and no significant loss in resolution was observed down to a pump 2/pump 1 flow ratio of 5. If higher flow rates are required in pump 1 to establish shorter residence times in the IMER, the flow rate in pump 2 must be concomitantly increased in order to maintain a pump 2/pump 1 flow rate proportion of at least of 5 for a subsequent highly efficient separation. Note that the system pressure limit becomes the boundary condition in this set up, which can in principle be easily solved by reducing the pressure drop in pump 1 through the use of larger particles, shorter columns or broader columns in the IMER configuration.

Figure 3-A and 3-B show the chromatograms of the 41-*mer* ON using a blank reactor (epoxy-silica deactivated with glycine) and the IMER, respectively. A flow rate of 50 µL/min was used in pump 1, and was maintained for 40 min in order to elute at least 4 IMER volumes. The effect of the IMER on the ON can be clearly observed, and the main degraded fragments can be resolved using this chromatographic technique.

Subsequently, the usage of an internal standard was explored as one potential application of this methodology is to perform quantitative degradation studies. Caffeine (1 mM) was used as internal standard (IS) as it demonstrated not to interact with the IMER, and also presented sufficient focusing in the methodology. However, the variation of the peak shape of the IS was noticeable at different residence times, and was not suitable for a focusing period which exceeded 6 times the IMER volume. By exceeding 4 times the IMER volume, it could be ensured that all the sample bands eluted from the IMER. On the other hand, this drastically extended the analysis time, particularly when long residence times were used. However, by comparing the area of the internal standard at different focusing times

and IMER residence periods, no significant differences on the peak areas were observed up to 1.5 IMER volumes. To deliver degradation profiles that do not only focus on the degradation of the main compound, the residence time was tuned to allow the proper detection of the degraded peaks which might not be detected if the activity of the reactor is too high as they also subjected to degradation. The 300 Å IMER was therefore employed for attaining the uncomplete degradations profiles presented in this paper, as if more active reactors are used the flow rate trough the IMER must be increased, what increases the system pressure and mobile phase consumptions.

3.3.2 Online IMER-IEC

It is known that IEC generates higher peak capacities and similar peak shapes for A, T, C , G and U homo-oligonucleotides in comparison to IPC, were ONs rich in C and G are poorly separated [29, 30]. Therefore, the hyphenation of the IMER with IEC was also investigated. The same experiments as in the IPC approach were performed in order to explore the focusing of the ONs in this IEC variant. The ONs focused without any problem, and flow rates ratios pump 2/pump 1 up to 2.5 were successfully tested. Higher flow rates (>200 µL/min) could be used in pump 1 without any consequences on the LC performance; nevertheless, they were not fully explored as the pressure drop in pump 1 almost reached its operational limits with the IMERs. Is important to point out that the pressure experienced by pump 2 with the 25 cm IEC column at 500 µL/min did not exceed 100 bar; however, the operational pressure limit of this column is 250 bar.

Figure 4-A and 4-B, depict the chromatograms of the 41-*mer* ON using a blank reactor and the IMER respectively. The same instrumental conditions were used as for the chromatograms in figure 3 with the exception for the injection volume, which was increased to allow for an improved detection. Adenosine monophosphate (AMP) was employed as IS (1 mM), and it demonstrated to focus in an excellent way, in

391 the same extent as the ONs. The higher peak capacity in the IEC methodology can be assessed after
392 comparing the chromatograms of figures 3 and 4. An important aspect to emphasize in the IEC
393 methodology is that the separation must be performed under denaturing conditions (pH>10.5) [30, 32],
394 as the reaction fragments can experience hydrogen bond interactions between them, which would lead
395 to unreproducible degradation profiles if the separation was to be performed at lower pH values.

396

397 *3.4 Stability evaluation of antisense ONs*

398 A number of natural and modified ONs were subsequently tested with the developed IMER-LC
399 methodologies for the evaluation of the performance of the method in stability assessments. The
400 degradation profiles were recorded for both methodologies (IMER-IPC and IMER-IEC) and comparisons
401 with the respective blanks were carried out.

402 The improved, but not complete resistance towards DNase I of the phosphorothioate modification is
403 visualized in figure 5, where plots A and B correspond to a T15 phosphodiester ON, while plots C and D
404 to a the phosphorothioate ON. It can be observed that after ~50 min of residence time in the IMER, the
405 T15 ON is practically completely degraded (plot B compared to its blank plot A), while the T15-
406 phosphorothioate ON only slightly degraded (plot D compared to its blank plot C). In the plots E to H the
407 specificity of the IMER towards DNA ONs is demonstrated, as the RNA ON passes intact through the
408 IMER (plots G and H).

409 Changes in the degradation profiles when injecting a mixture of ONs demonstrate the applicability of the
410 methodology for footprinting analysis and/or cleavage protection carried out by a hybridization process
411 for instance. Hybridization between the A30 and T30 ONs was tested for that purpose. The differences
412 in the degradation profiles are visualized in figure 6, where plot A corresponds to the analysis of a (T30,

A30) ON mixture in the blank IMER, while plots B, C and D depict the degradation profiles of a single stranded A30 ON, a single stranded T30 ON and of the (A30, T30) ON duplex, respectively.

Moreover, similar ON degradation profiles consisting mainly of nucleotide deletions form the main compound as observed in figure 6 have been observed in vivo [31] and in vitro metabolism studies of ONs [23, 24, 31] as well as for small interfering RNA (siRNA) [20-22]. This proves the utility of the developed methodologies as no sample preparation methodologies are required previous to the instrumental analysis. Furthermore, under this approach the usage of only one enzyme can be extended to multiple enzymes to deliver more representative information on the metabolism of this molecules.

One of the advantages of the usage of IMERs is their stability and extended life time. Indeed, the IMERs demonstrated to be highly active even up to 3 months after their construction, a period during which they were subjected to analysis and storage conditions (4 ℃). Figure 7 shows the degradation profile of a (T30, A30) ON mixture tested on the 300 Å IMER after 3 months from its manufacturing. Furthermore, the high reproducibility of the methodology can also be visualized in figure 7, where the degradation profiles obtained during three consecutive days are presented. Additionally, the relative standard deviation (RSD) on the area of selected product peaks is also displayed. The %RSD did not exceed 15%, and such high values, have been only been observed for the smallest product peaks, while for larger product fragments the RSDs were around 5%. The average %RSD in all cases was below 10%, which is exceptionally good as it was measured on the generated degradation fragments, demonstrating the potential of the methodology for performing online quantitative degradation studies. Note that the %RDS on the retention times were below 0.1% for all the cases.

The limits of detection (LOD) of the IMER-LC methodologies are determined by the signal to noise ratio (S/N=3) [64]. The calculated LOD values for the 12-*mer* and the 41-*mer* ONs are shown in table 3. Two LOD values were determined, one corresponding to the instrumental LOD, determined by injection of

436 the ONs trough a blank IMER, and the IMER-LC LOD, which was determined using the IMER. This last
437 LOD, was calculated based on the product fragments rather than by the main peak, and was defined as
438 the concentration where at least 80% of the fragments in a degradation profile were detected with a
439 S/N=3. This LOD is dependent on the residence time, as the number and intensity of the generated
440 fragments are strongly influenced by it. Nevertheless, this LOD provides a useful insight for setting on
441 analyses with this setup. In general, somewhat higher LODs were observed for the IMER-IEC
442 methodology when compared to the IMER-IPC, and were attributed to the higher signal noise ratio
443 generated by the IEC mobile phase containing 1.25 M NaCl.

444

445 **4. Conclusion**

446 An online IMER-LC platform for the stability evaluation of antisense DNA ONs was developed. IPC and
447 IEC were successfully coupled with a DNase I IMER. More in detail, the IMER-IEC method presented a
448 higher resolving power for the ON degradation products when compared to the IMER-IPC method. On
449 the other hand, the latter allows for easier hyphenation with mass spectrometry [29, 65]. In addition to
450 the extended life time of the IMER, both methodologies showed a high reproducibility in area and
451 retention time. This platform demonstrates potential for application in laboratories where stability tests
452 of ONs are often performed. Moreover, the construction of IMERs with other endonucleases and
453 exonucleases, specific or not to the sugar and their coupling to LC should also be explored, as this would
454 cover stability assessments of a much wider range of ON modifications. Furthermore, the construction
455 of an IMER containing multiple nucleases, based either on the simultaneous immobilization of various
456 nucleases to a support or by the interconnection of IMER subunits is promising, as many nucleases share
457 the same activation conditions by divalent cations such as Ca^{2+} and Mg^{2+}, allowing for an easy
458 hyphenation with LC. Implementation of the here described methodology for nuclease resistance

assessment will allow generation of uniform and reliable data, finally enabling reliable comparison of different approaches towards enhancement of nucleic acid stability, which is of prime importance in the fast developing field of ON based drug development.

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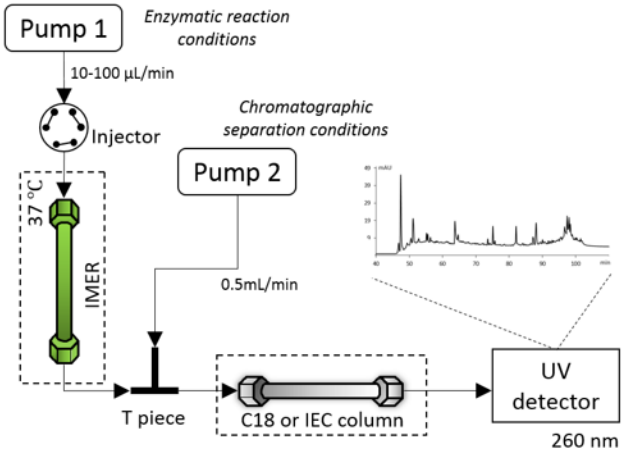

Figure 1. Schematic representation of the IMER-LC setup.

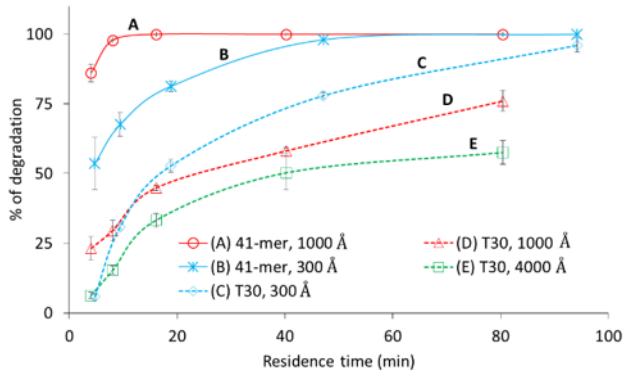

Figure 2. Oligonucleotide degradation in the the 300 Å, 1000 Å, 4000 Å IMERs. A T30 ON and the 41-*mer* ON are compared. The degradation percentage is plotted against de residence time in the IMER. The confidence interval (α=0.05, n-1 d.f.) of the mean is plotted for each point.

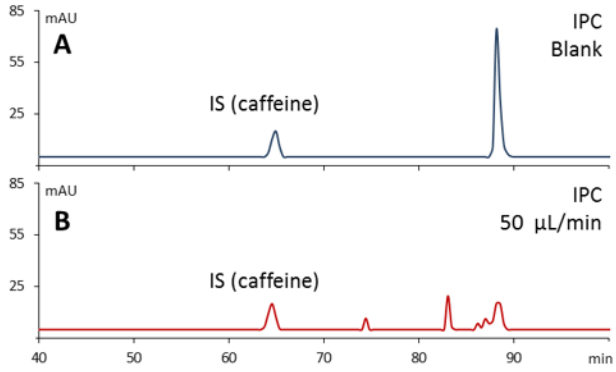

Figure 3. IMER-IPC chromatograms of a 41-*mer* ON. A) blank IMER; B) DNase I IMER. ~10 min of residence time in the 300 Å IMER (Flow rate of pump 1 = 50 µL/min). The sample was allowed to focus for 40 min before the LC gradient start. IS: internal standard. Injection: 1 µL of a 100 µM ON solution.

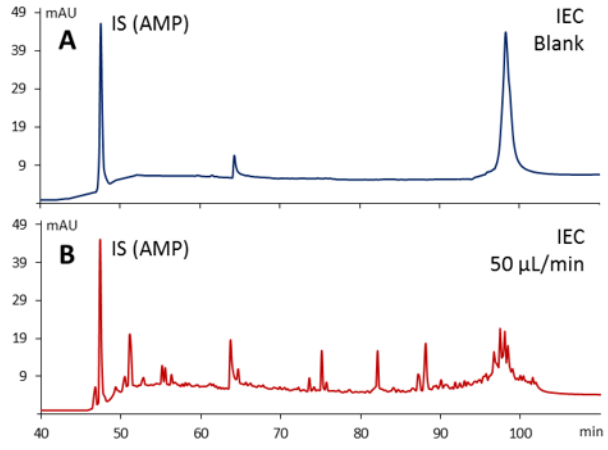

Figure 4. IMER-IEC chromatograms of a 41-*mer* ON. A) blank IMER; B) DNase I IMER. ~10 min of residence time in the 300 Å IMER (Flow rate of pump 1 = 50 µL/min). The sample was allowed to focus for 40 min before the LC gradient start. IS: internal standard, adenosine monophosphate (AMP). Injection: 3 µL of a 100 µM ON solution.

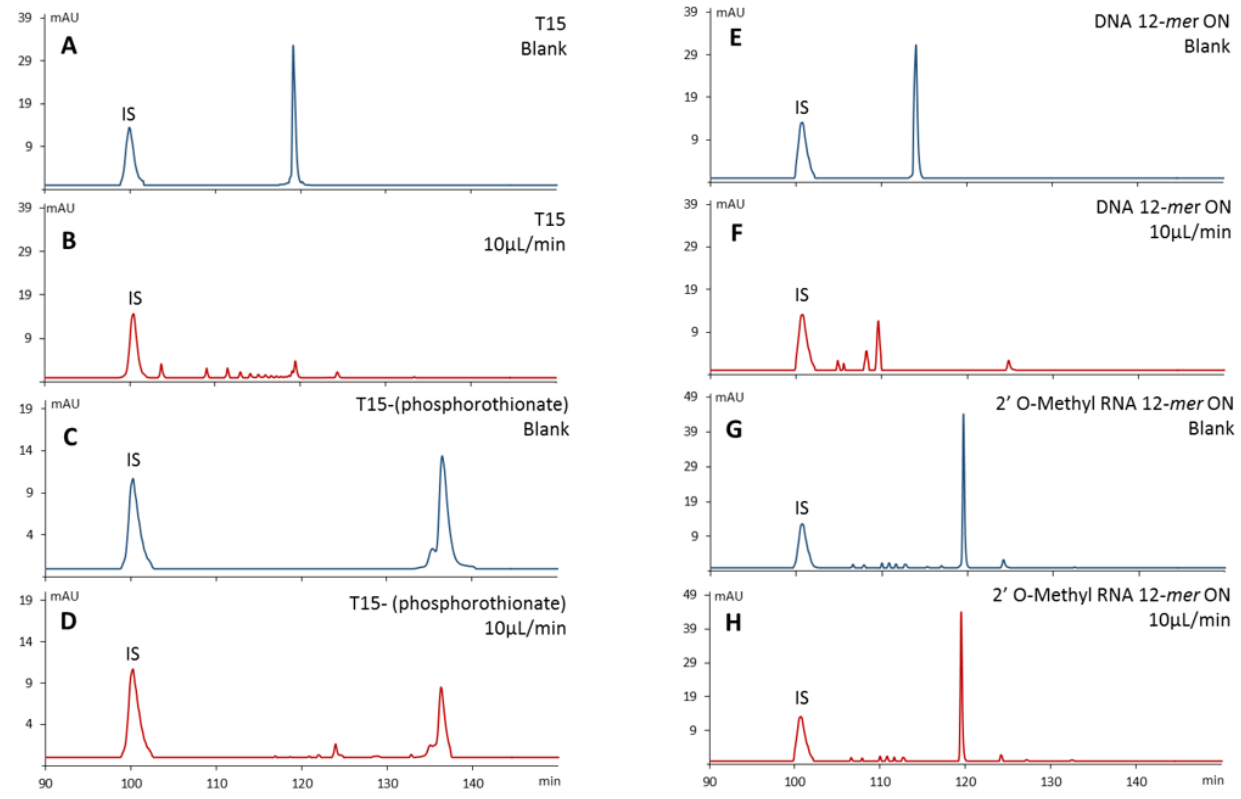

Figure 5. IMER-IPC chromatograms. The analyzed ON sample is indicated for each chromatogram. A), C), E) and G) correspond to a blank IMER, while B), D), F) and H) to the DNase I IMER. ~50 min of residence time in the 300 Å IMER (Flow rate of pump 1 = 10 µL/min). The sample was allowed to focus for 80 min before the LC gradient start. IS: internal standard, caffeine 1 mM. Injection: 1 µL of a 100 µM ON solution.

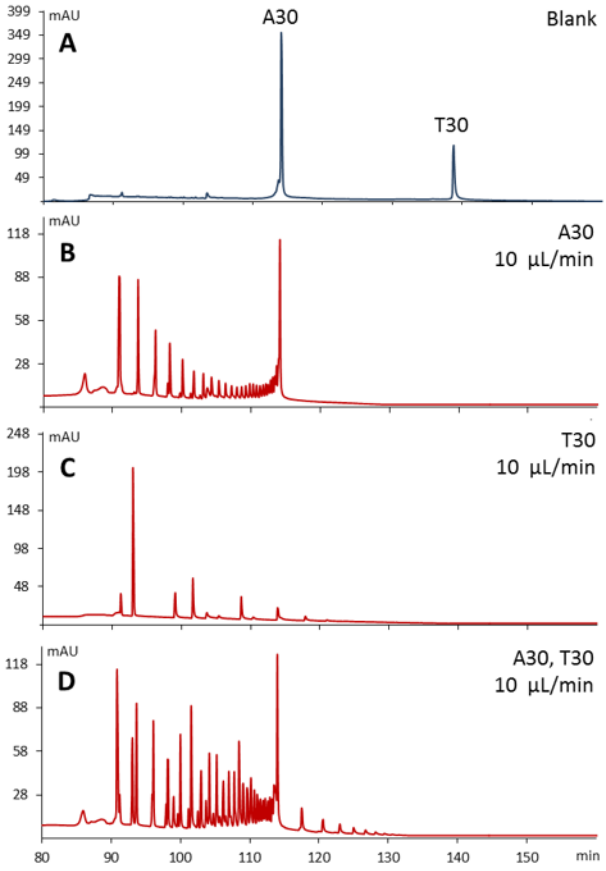

Figure 6. IMER-IEC chromatograms. The analyzed ON sample is indicated for each chromatogram. A) corresponds to a blank IMER, while B), C) and D) to the DNase I IMER. ~50 min of residence time in the 300 Å IMER (Flow rate of pump 1 = 10 µL/min). The sample was allowed to focus for 80 min before the LC gradient start. Injection: 10 µL of a 50 µM ON solution. Each ON is present at the same concentration. No internal standard was employed.

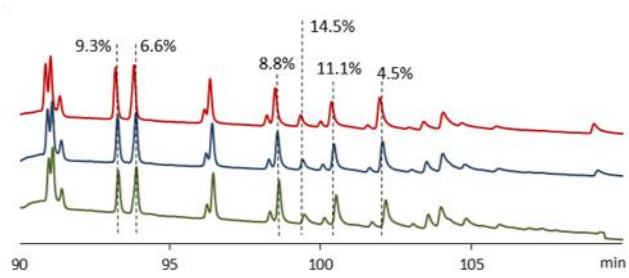

Figure 7. Three IMER-IEC chromatograms of a (T30, A30) ON mixture analyzed during 3 consecutive days in a 3 months old 300 Å IMER. The %RSD on the area is indicated for few peaks. ~50 min of residence time in the IMER (Flow rate of pump 1 = 10 µL/min). The sample was allowed to focus for 80 min before the LC gradient start. Injection: 5 µL of a 50 µM ON solution. No internal standard was employed.

Table 1. Chromatographic conditions of the IMER-LC setup.

Flow rate (µL/min)			
Pump 1	Pump 2 (Eluent)	Time (min)	Event
50	500 (100% A)	0.00-40.00	(i) Elution of the analytes trough the IMER and focusing in the analytical column
1	500 (Gradient A-B)	[a]40.01-90.00 [b]40.01-130.00	(ii) Separation of the analytes by the analytical column

[a] Ion-pair chromatography (A: 100 mM TEA, pH 5.5; B: ACN)
[b] Ion-exchange chromatography (A: H_2O, pH 11.5; B: 1.25 NaCl, pH 11.5)

Table 2. Properties of the immobilized DNase I silica particles

Silica pore size	Pore volume (mL/g)	Immobilized DNase I (mg/g)[a]	Activity/support (pmol T30/min·g)
300 Å	0.8	8.2 ± 0.3	123.8 ± 42.5
1000 Å	0.8	6.1 ± 0.2	643.3 ± 156.0
4000 Å	0.7	4.9 ± 0.1	361.8 ± 23

5 µm silica particles where employed.
The free DNase I used for immobilization has a specific activity of 6.09×10^5 pmol T30/min·g
[a] Result expressed as the mean ± $CI_{(\alpha=0.05)}$ (n=3).

Table 3. Limit of detection for the LC methodologies and the IMER-LC methods.

Compounds	LOD Main peak[a]		LOD Product fragments[b]	
	IPC	IEC	IMER-IPC	IMER-IEC
12-*mer* ON	0.01	0.11	0.18	1.1
41-*mer* ON	0.03	0.12	0.63	1.3

The values are given in µg/µL concentrations and considering an injection volume of 1 µL.
Only DNA based ON were tested.
[a] Determined with a blank IMER, S/N=3.
[b] Determined under a flow rate of 50 µL/min trough the 300 Å IMER and considering a detection of 80% of the product fragments with a S/N=3.